Supplementary Material

Leveraging Synthetic Imagery and YOLOv8 for a Novel Colorimetric Approach to Paper-Based Point-of-Care Male Fertility Testing

Materials and Methods

Reagents and materials: Whatman chromatography paper no. 1, 240 mm was purchased from GE (1001-042, UK). bromothymol blue (MKCK0744), bromocresol green (MKCM6662), thymol blue (MKCD1057), sucrose (BCCF1187), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (MKCN5605), ∝-chymotrypsin (SLCH1926), sodium citrate tribasic dihydrate (6132-04-03), magnesium chloride ($MgCl_2$, 7786-30-3), bovine serum albumin (BSA, 9048-46-8), phosphate buffered saline (LB.SA.P4417-100TAB), sodium hydroxide (NaOH, 1310-73-2), hydrochloric acid (HCl, 7647-01-0) and glycerol solution (49782) were received from Sigma Aldrich (Germany). 2 mL microcentrifuge tubes (MTPPN21020002), polypropylene & polycarbonate vacuum desiccator (I.039.12.250), pH 4 buffer solution (LR3331310ANW), pH 7 buffer solution (LR3341308ANW), pH 10 buffer solution (LR3361309ANW), hemocytometer (075.03.002), sodium chloride (NaCl, LR1441312APW), sodium sulfate anhydrous (Na_2SO_4, LR1601304ANW), and potassium chloride (KCl, LR1091301ANW) were obtained from ISOLAB, Turkiye. double-sided tape, glucose (152300134), calcium chloride dihydrate ($CaCl_2$, ZK.100470.1000), and DI water device (MP minipure dest mini) were bought from Nippon, Edukim, Zag Kimya, medikal endüstriyel sistemler, Turkiye, respectively. Semen sample tubes 2 mL with breakable cap was purcehased from Qeak Lab, China. The healthy semen samples were collected from Koç university hospital, Turkiye.

Design and manufacturing of the assay

The reference color paper and the shape of the cellulose test paper was designed on Adobe Illustrator. Reference color papers were printed on two-side coated glossy paper with a regular laser printer. The coating of the glossy paper increased the hydrophobicity of reference color paper reducing the undesired spread of the sample. The flower cellulose paper was cut using EpilogLaser (USA) Fusion Maker desktop laser machine. To assemble the test paper to the reference paper, a commercial double-sided tape was cut in the same shape. Printer speed and power were set to 95% and 5% for cutting cellulose paper while the values were adjusted to 90% and 10% for cutting the thicker double-sided tape. These values allowed for the

achievement of a complete cut without burning the paper or the tape. Later, the double-sided tape was pasted onto the reference color paper by hand. Then the cellulose test paper was carefully aligned and assembled on the open end of the tape. The hydrophobic structure of the double-sided tape further elevated the capacity of the test to hold the sample on cellulose test paper.

Effectiveness of laser cutting

We achieved much better results using the laser cutting method compared to the studies carried out with the traditional hydrophobic marker plotting method, and thus we prevented semen leakages. Thanks to the laser cutting method, leakages in plot drawings were eliminated. In the experiments carried out with the semen samples, it was observed that the semen passed through the ink drawn with the pencil plotter and disrupted the channels. Also, in some cases, the test points were not evenly distributed with the pencil plotter since the surfactant molecules in the semen samples dissolved the ink and passed through the barriers. As a solution to this, instead of drawing with ink, the channels were created by precisely cutting the paper directly with a laser cutter. Double-sided tape was also cut in the same way to create a hydrophobic barrier on the paper. Similarly, tests were constructed in this way, and no leakage or flow was observed. The illustration of the final design was given in the supplementary material (Fig. S1).

Sucrose treatment

Preparation of a 5% sucrose solution is the first step in the experiment. For this purpose, distilled water was used to dissolve a precise mass of sucrose, ensuring the homogeneity of the solution. Whatman papers are dipped into a 5% sucrose solution in petri dish and allowed to absorb the solution through capillary action (Tsao et al., 2021). The purpose of this step is to uniformly impregnate the papers with the sucrose solution. Filter papers infused with sucrose were gently removed from the sucrose solution once they were sufficiently saturated. A vacuum desiccator was used to accelerate the elimination of extra moisture and to make it easier for sucrose to be distributed evenly throughout the paper matrix. The Whatman papers were allowed to air dry inside of this desiccator at reduced pressure, which effectively hastened the evaporation of water from the papers. To ensure that all moisture has been removed, this drying process was carefully observed. Once fully dried, papers were taken out of the desiccator to be positioned on the center of color barcode and secured in place with double-sided tape.

pH indicators and MTT preparation

Four different indicator solutions were prepared: Bromocresol Green (BG), Bromothymol Blue (BB), Thymol Blue (TB), and MTT. The BB, BG, and TB solutions should be stored at room temperature in the dark, while the MTT solution should be kept at +4°C and protected from light. For the BG solution, a specified amount of BG was dissolved in a diluted NaOH solution. Similarly, BB was prepared in the same NaOH solution, while TB was dissolved in a lower concentration of NaOH. Finally, a measured amount of MTT was dissolved in distilled water to create a stock solution (Buranaamnuay, 2021; Nasr-Esfahani et al., 2002).

Microfluidic test

There are 8 separate sensing circles on the test paper, where each of the four reagent solutions is immobilized in duplicate. 1 μL of each reagent solution was added to the detection circles using a micropipette: BG, TB, BB and MTT in two circles, respectively.

Viscosity assay

To optimize flow dynamics in the context of paper-based microfluidics, we investigated the relationship between semen viscosity and flow duration while determining the optimal concentration of a liquefying agent. To simulate semen under controlled conditions, glycerol was employed as a model fluid in viscosity experiments. Various glycerol-to-water ratios (Table S1) were prepared and tested, with flow timing initiated upon each application (Cheng, 2008; Volk & Kähler, 2018). Results (Fig. S3A) revealed a linear correlation between viscosity and flow duration, highlighting the critical influence of viscosity on sample flow characteristics.

Subsequently, experiments with actual semen samples of two different viscosities were conducted to further refine this optimization. Chymotrypsin concentrations ranging from 20% to 60% were applied to evaluate their liquefying efficacy. The flow duration, measured from the point of application until the sample fully traversed the test paper, indicated that a 30% chymotrypsin concentration achieved an optimal balance between liquefaction efficiency and flow consistency (Fig. S3B). Repeatability of this optimal concentration was confirmed through four independent trials using semen samples (Fig. S4).

This assay provided key insights into the rheological behavior of semen and analogous fluids under varying viscosity conditions. The findings not only enhance our understanding of viscosity's role in optimizing flow but also offer practical guidance for the design and implementation of paper-based microfluidic systems.

Semen preparation

The semen sample was allowed to thaw at room temperature while an ∝-chymotrypsin solution was prepared by dissolving α-chymotrypsin in PBS. Once the semen sample reached a liquid state, 30% of the chymotrypsin solution was added to the semen sample to enable liquefaction Since 4 drops of the total sample will be used for the test, the total concentration of the semen sample and liquefier should be at least 75 µL. The mixture was gently agitated, and after a 10-minute incubation, a portion of the liquefied sample was placed at the center of the sample spot for further analysis (Schallmoser et al., 2020). After 10 minutes, images were taken using a smartphone camera to analyze the semen quantitative assessment.

Sperm count and pH assessment

39 images with varying pH levels (5< pH <7, 7< pH <8, 8< pH <10) and sperm counts (sc >10M, sc <10M) is obtained to assess resulting colors and flow patterns in sensing regions. We explored YOLOv8 by Ultralytics as a single-step solution for both ROI extraction and classification using colorimetric data. Unlike traditional methods requiring cascaded image processing algorithms, YOLOv8 offers efficient real-time object detection and classification (Kang & Kim, 2023), making it suitable for colorimetric mobile phone biosensor sensing applications. However, obtaining a labeled dataset for training presented a challenge due to sample scarcity and dedicated lab procedures for obtaining spermiogram data. To address this, we implemented a procedural image generator that captured variations in image quality, flow patterns, color shifts, and lighting conditions to mimic actual mobile phone images. We then fine-tuned YOLOv8 model to segment and label the region of interests (ROI) of preprocessed real samples.

To evaluate the performance of the proposed single-step solution, we prepared two datasets. The first, a well-prepared test dataset, includes 39 images generated from the same solutions used to fine-tune the YOLOv8 model. In this dataset, sperm count and pH values were measured using gold-standard laboratory equipment, with solutions applied according to the protocol detailed in the *Semen Preparation* section.

The second dataset presents a more challenging scenario. It comprises 228 artificial samples with varying pH ranges, where pH values were measured using standard urine dipstick tests. For this dataset, the designed instrument interfaced with the artificial samples without following a standardized protocol. Additionally, images were captured under inconsistent lighting conditions, often within a short timeframe, leading to variability in image quality.

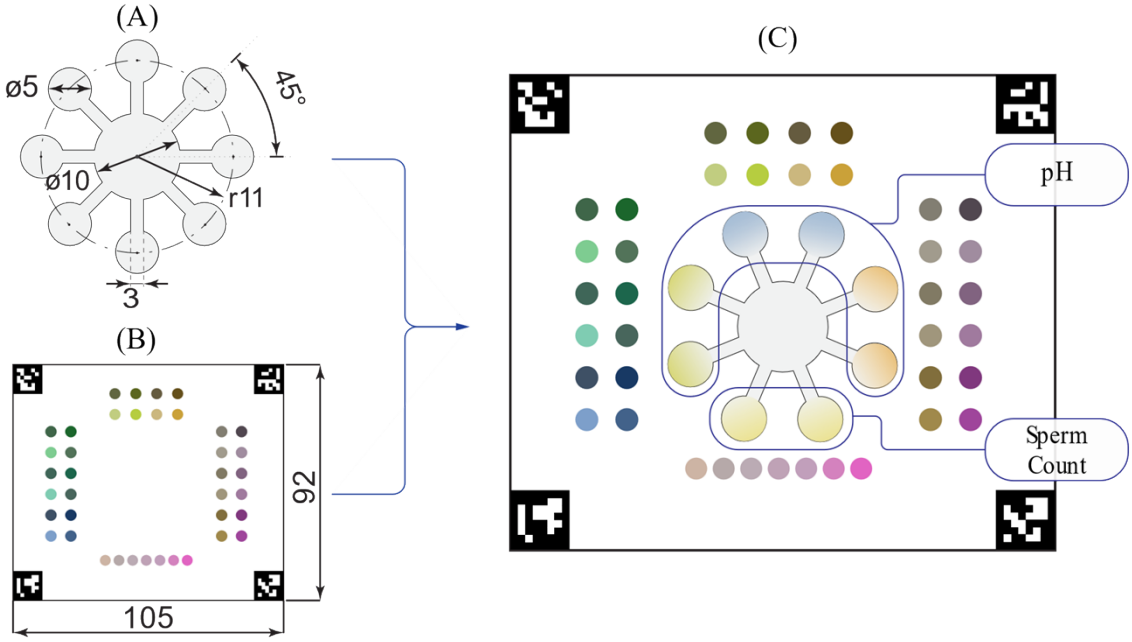


Fig. S1. Dimensions of the reference color paper (A) and cellulose paper (B) of the assay. (C) The cellulose paper was bonded to the reference paper with a laser cut double-sided tape of same shape. MTT and pH indicators were dropped to respective regions of the assay. All dimensions are given in millimeters. r: radius; Ø: diameter.

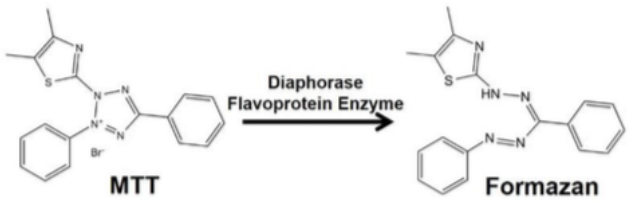


Fig. S2. Conversion of the MTT reagent to colored product (Peter Brescia, 2021).

Table S1: Calculation of viscosity of glycerin used to mimic sperm

Sample No:	1		2		3		4		5	
Substance	Water	Glycine	Water	Glycine	Water	Glycine	Water	Glycine	Water	Glycine
Volume (mL)	3,00	2,50	2,50	2,50	2,00	2,50	2,00	3,00	2,00	3,50
Dynamic viscosity of mixture [Ns/m^2]	0,0053		0,0068		0,095		0,0127		0,0164	
Duration time (min)	3		3.45		4		5.15		6.15	

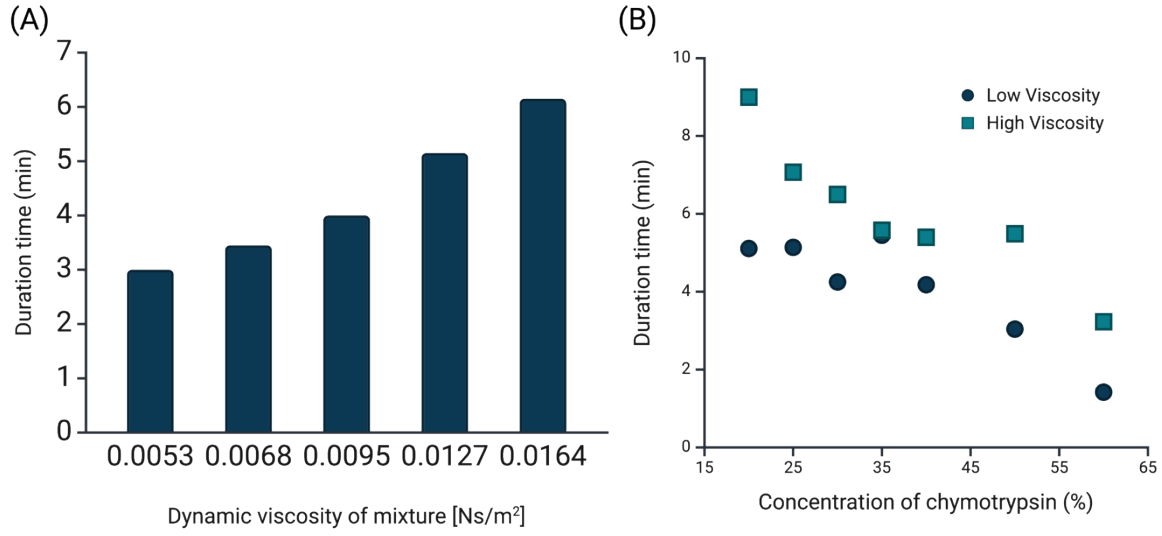


Fig. S3. Viscosity assay. (A) The bar graph presents the impact of different dynamic viscosities (0.0053 to 0.0164 Ns/m²) on the duration time of the test response, indicating that higher viscosity prolongs the reaction time, (B) The plot comparing the effects of chymotrypsin concentration on duration time under low and high viscosity conditions, indicating a possible interaction effect

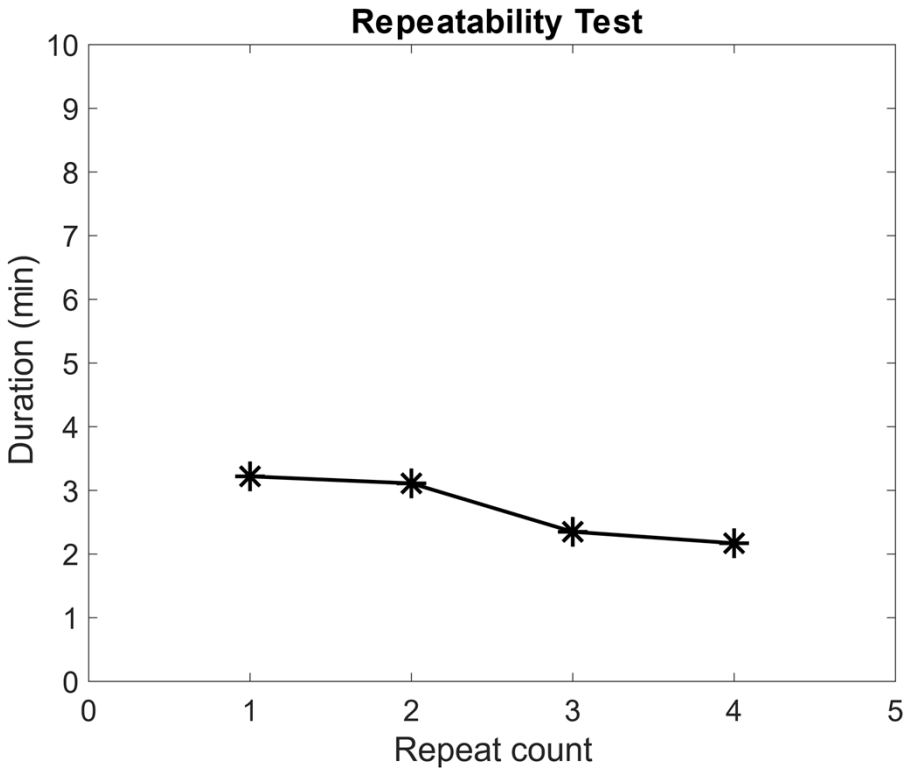


Fig. S4. Repeatability testing with selected 30% liquefier using semen sample 2 **(dilute semen sample)**; the vertical axis shows duration time in minutes, while the lateral axis indicates the repetition numbers.

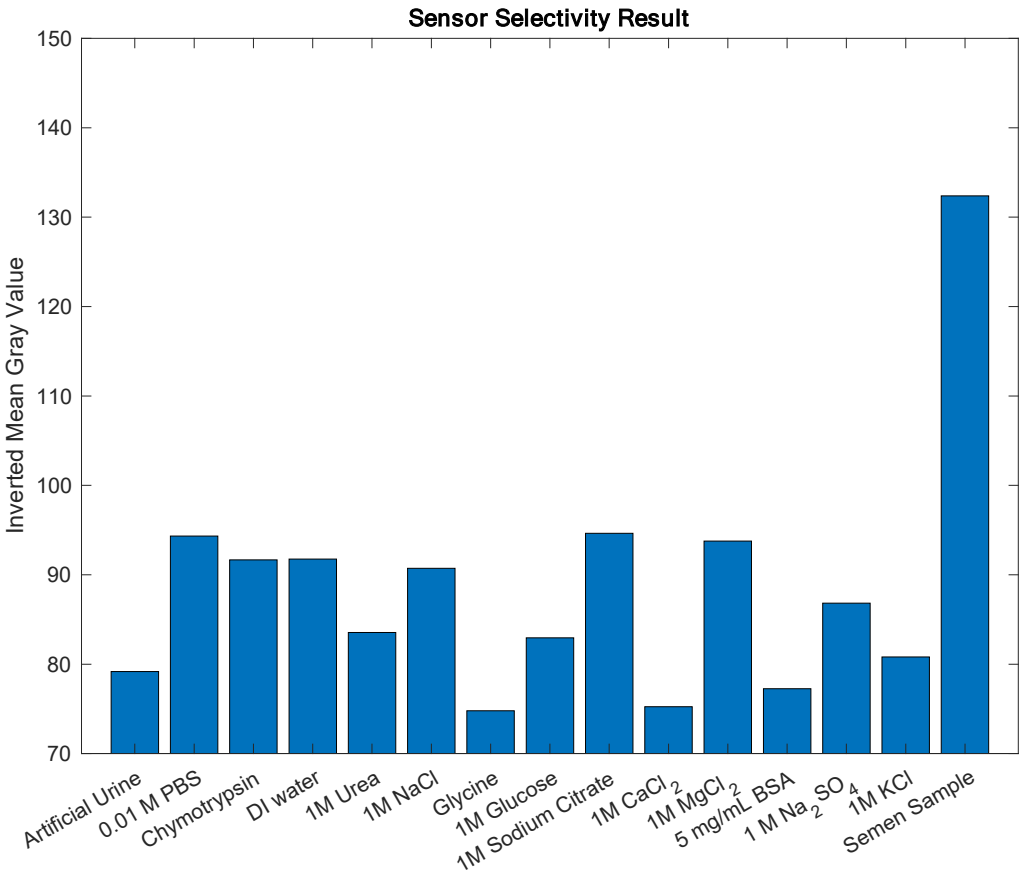


Fig. S5. Selectivity assay results without blank correction.

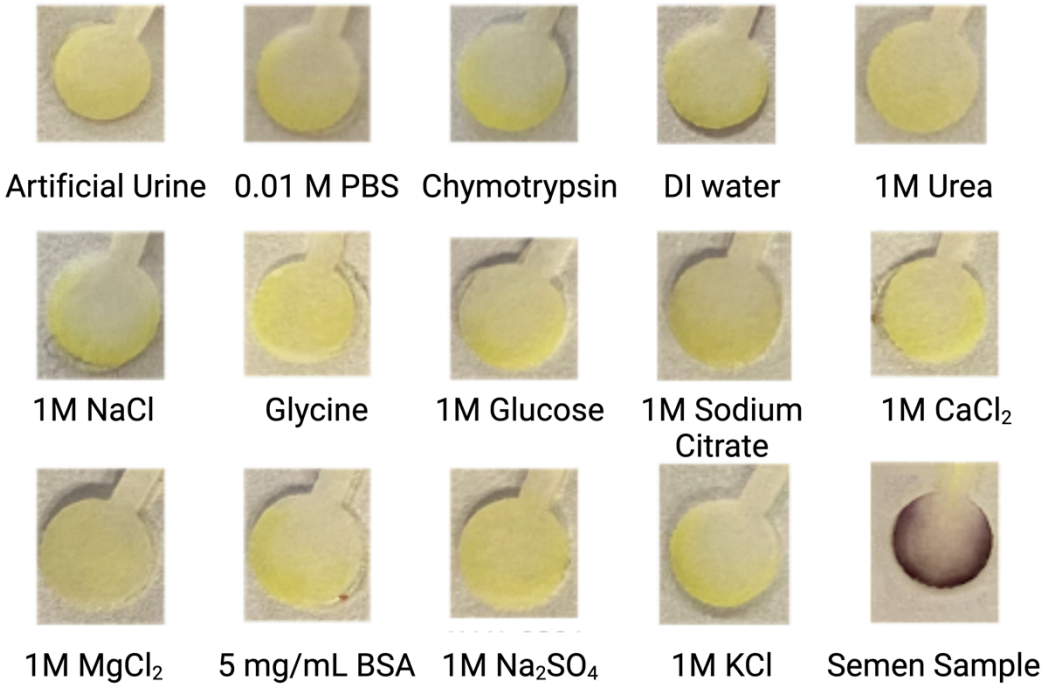


Fig. S6. Results of the selectivity assay, raw data.

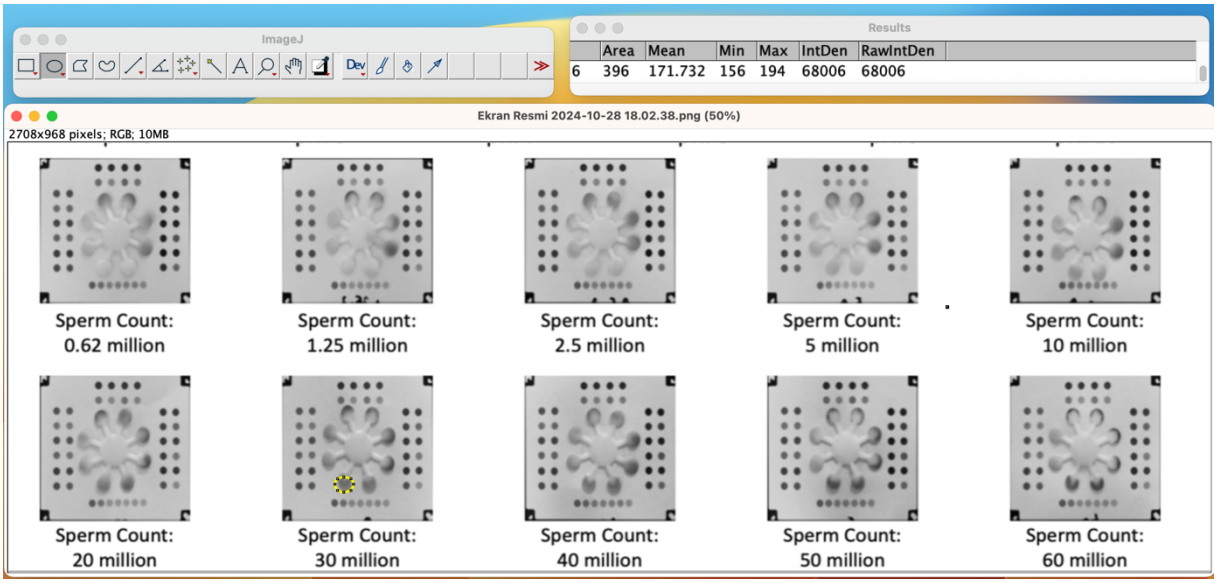


Fig. S7. Mean gray value measurement using ImageJ (8-bit image).

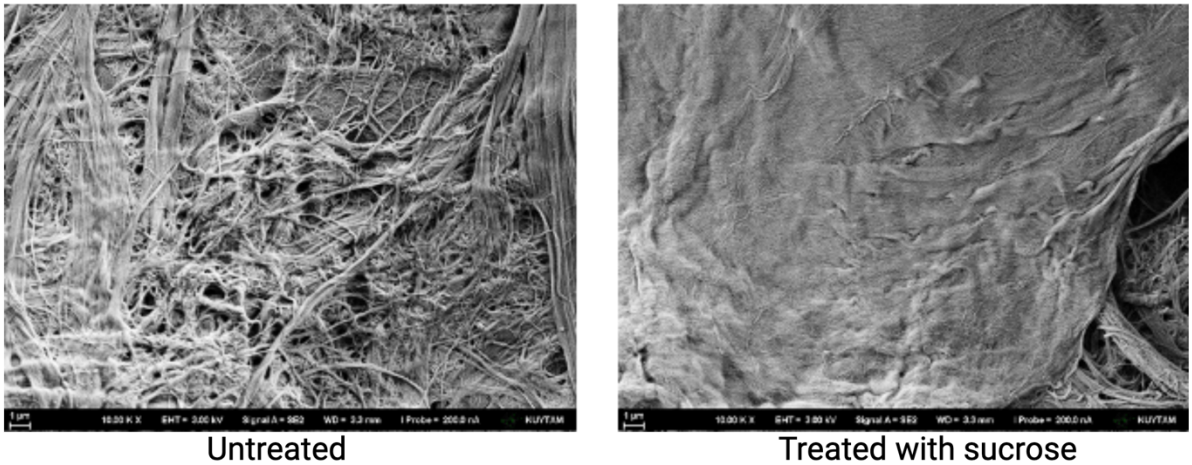


Fig. S8. SEM images of sucrose effect on Whatmann Paper.

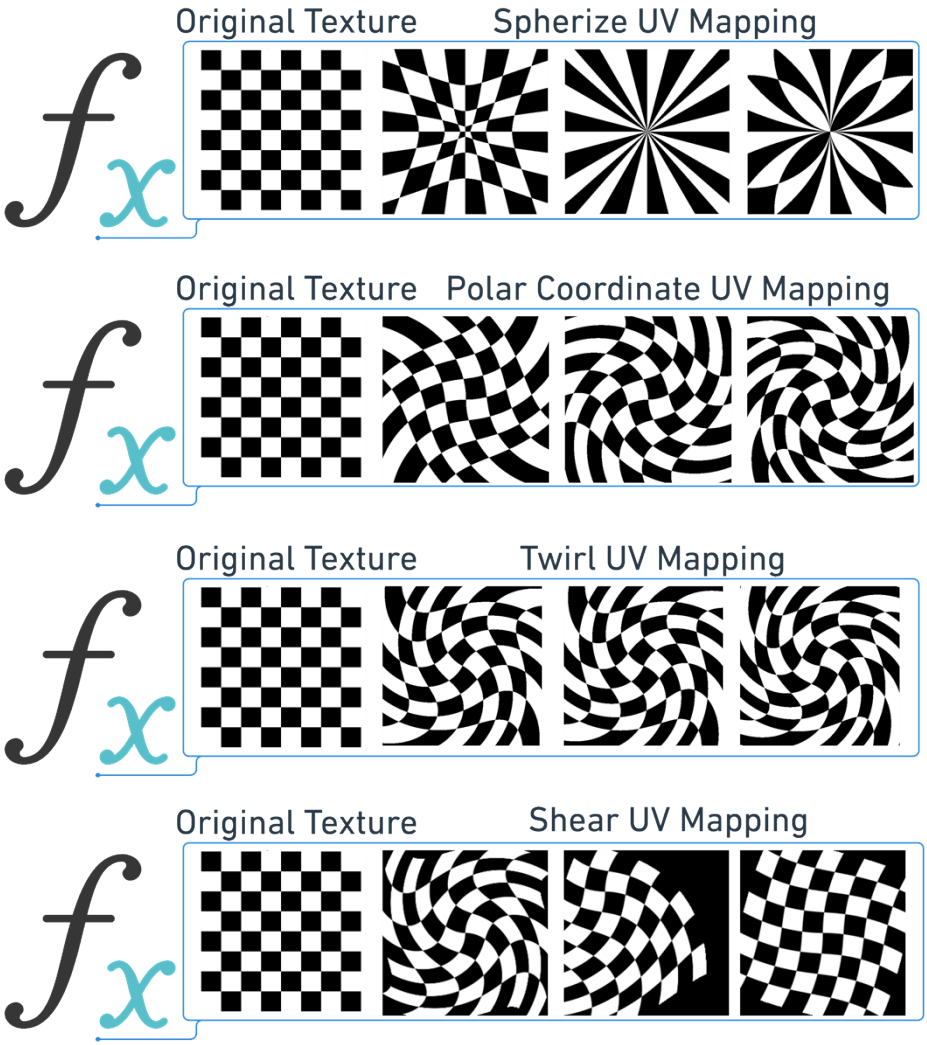
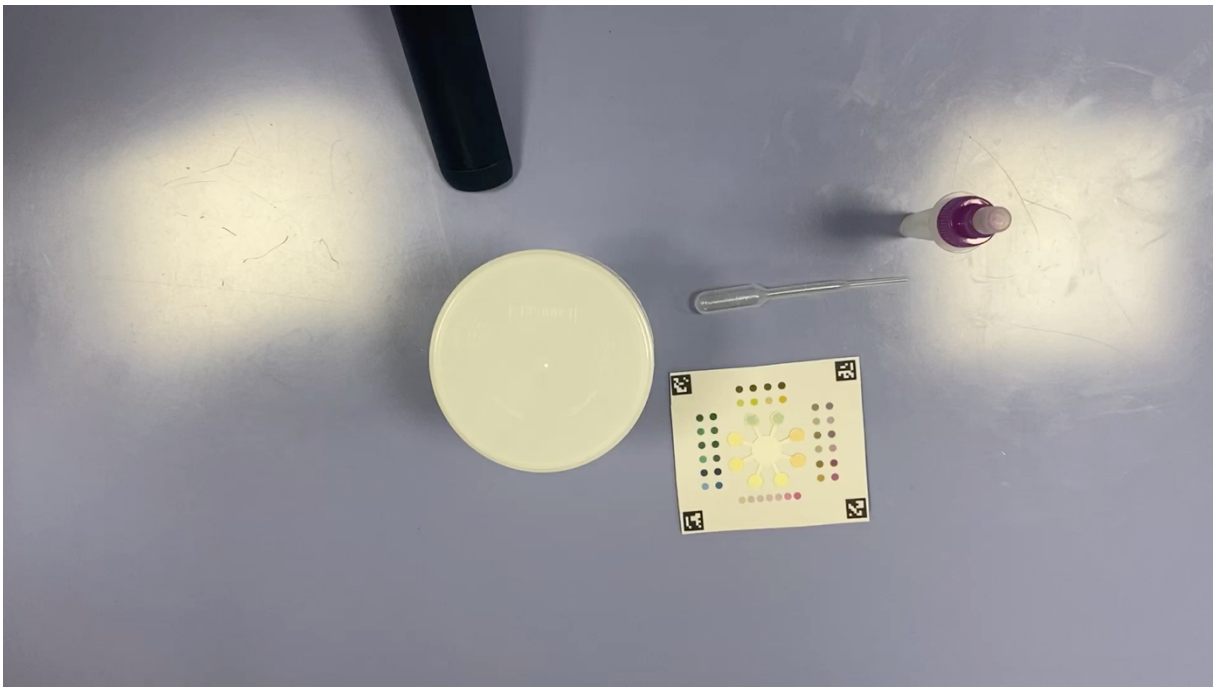
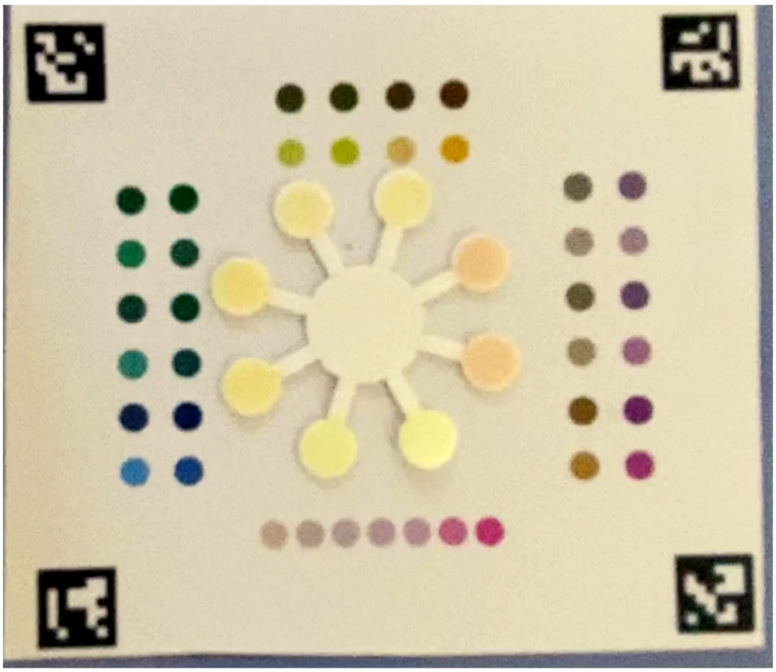


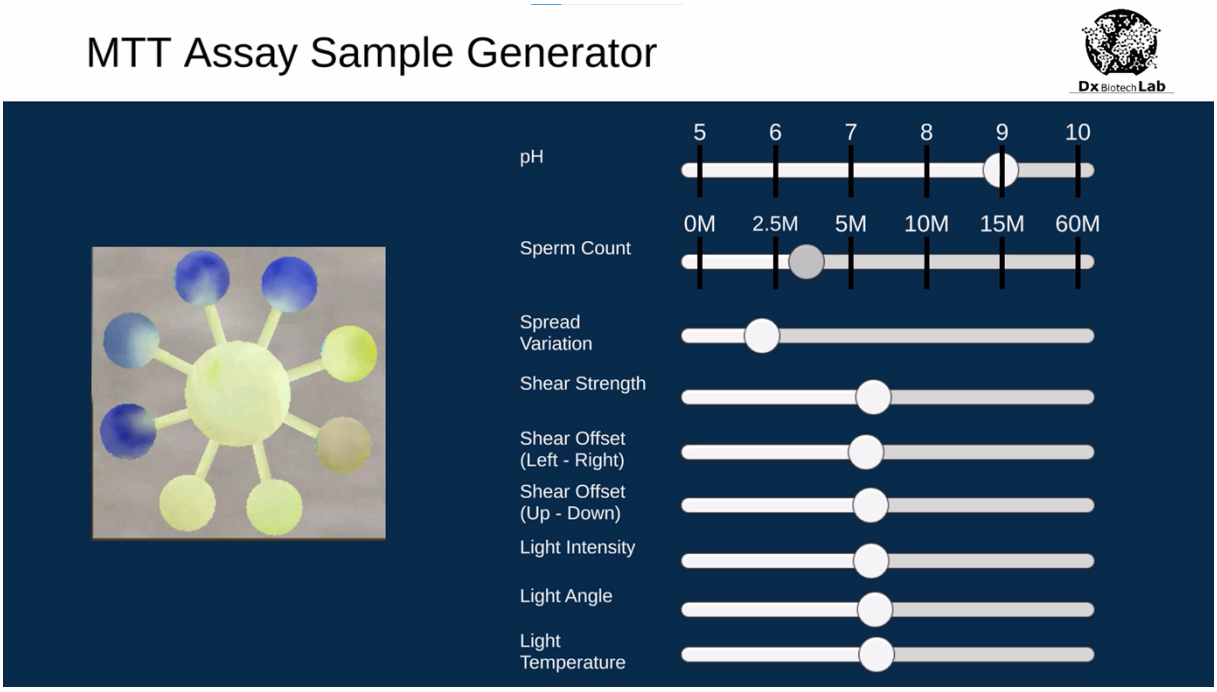
Fig. S9. Effect of various UV mapping functions on given original texture.



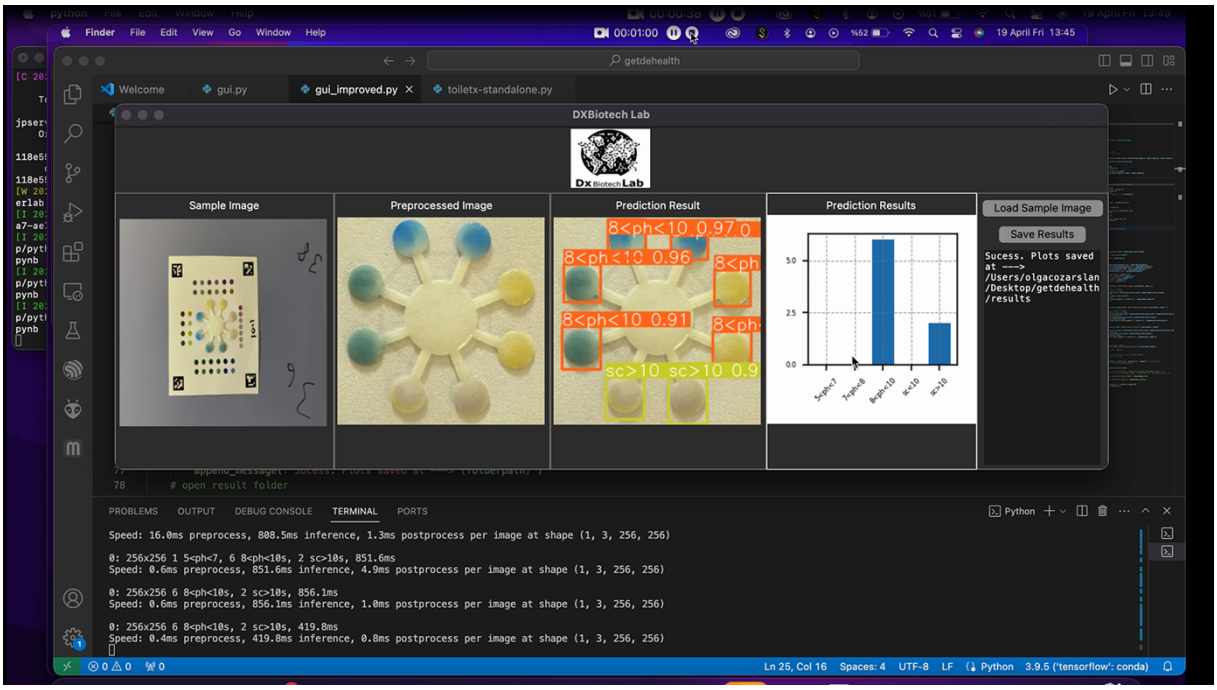
Movie S1. This video figure illustrates the usage process of a paper-based sperm sensor. Initially, semen collected in an ejaculatory cup is taken using a Pasteur pipette and transferred to a liquefying dropper. A ten-minute waiting period is applied to complete the liquefaction process. Subsequently, four drops of liquefied semen are applied to the test area, followed by a fifteen-minute incubation period to obtain the test result. This process provides a rapid, cost-effective, and accessible method, based on color changes indicative of sperm concentration and motility, offering significant contributions as a potential tool for assessing male reproductive health.



Movie S2. This figure shows a time-lapse video showing the process of semen being dripped from the dropper onto the test until complete dispersion. The accelerated video demonstrates the flow of semen across the test area, depicting how it spreads and interacts with the paper-based sensor. This visual representation provides insight into the dynamic behavior of semen as it moves and distributes on the test, aiding in understanding the kinetics of the interaction between the semen sample and the sensor components



Movie S3. This video showcasing the synthetic MTT assay generation tool we implemented. By adjusting the parameters, we can mimic the spatial information encoded in synhtetic region of interests with known ph and sperm count values.



Movie S4. This video showcases the automated workflow for obtaining the results from image captured with mobile phone camera. With this internal tool, our collaborators can see the results of the paper interfaced with semen sample.

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